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Evaluation of nootkatone in the antibacterial activity and inhibition of the efflux pumps NorA and MepA in *Staphylococcus aureus* strains

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ABSTRACT

Microorganisms naturally exist in the environment and live on humans as part of the normal microbiota. However, multidrug-resistant bacteria, which cause numerous infections and deaths annually, are a significant concern. Nootkatone, a compound known for its antibacterial, anti-inflammatory, anticancer, and antioxidant properties, is used in various industries. This study aimed to evaluate the antibacterial effects of nootkatone on *Staphylococcus aureus* strains, including those with efflux pump proteins NorA and MepA. Using the broth microdilution method, the minimum inhibitory concentration (MIC) of nootkatone was determined both alone and in combination with antibiotics norfloxacin, ciprofloxacin and with standard efflux pump substrate the ethidium bromide (EtBr). Nootkatone demonstrated intrinsic antibacterial action against the *S. aureus* K2068 strain with an MIC of 203 μg/mL, but no activity against *S. aureus* ATCC 29213 and *S. aureus* 1199. When combined with norfloxacin, it significantly reduced the MIC of the antibiotic against *S. aureus* ATCC 29213 and *S. aureus* 1199. With a significant increase in fluorescence against *S. aureus* 1199, nootkatone also showed potential as an inhibitor of the NorA efflux pump. The results suggest that nootkatone may inhibit efflux pumps, thereby enhancing the effectiveness of antibiotics. Further molecular studies are needed to fully understand its antibacterial mechanisms.

1. Introduction

Microorganisms can colonize various environments, including air, soil, and water. Bacteria are found acting commensally in the normal human microbiota, being present on the skin, in the oral cavity, and in the respiratory, intestinal, genital, and urinary tracts. In addition to their commensal form, they can act as pathogenic organisms causing infections (Novak Babič et al., 2020; Martínez et al., 2021). Bacterial infections occur due to factors such as the disruption of the epithelial barrier, imbalance of the immune system, and the normal microbiota, leading to the accelerated multiplication of pathogens and their colonization in previously sterile organs (Carvalho et al., 2021; Cruz-López et al., 2023).

Multidrug-resistant (MDR) bacteria are the main cause of infections in hospital settings, leading to increased morbidity and mortality among patients and posing a significant threat to global public health. MDR bacteria have acquired drug resistance after being selected through the inappropriate and excessive use of antibacterial drugs. As a result, conventional treatment with first-line antibiotics becomes ineffective against bacterial infections (Makabenta et al., 2021; Manohar et al., 2020; Su et al., 2022). These resistance mechanisms are characterized as an evolutionary phenomenon, which can be natural and intrinsic or acquired (Santana et al., 2023).

In this context, *Staphylococcus aureus* is a Gram-positive bacterial strain, primarily found on the skin and in the nasal mucosa. It stands out as a commensal bacterium, an opportunistic pathogen, and of high

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clinical importance due to its demonstrated resistance mechanisms, leading to difficult-to-treat infections and consequently causing severe diseases and deaths. The main resistance mechanisms in *S. aureus* strains involve alteration of the antibiotic target through mutation or addition of chemical groups, modification or inactivation of the antibiotic by enzymes, reduction of cell membrane permeability through negative regulation of porin expression, and efflux pump systems (Gaurav et al., 2023; Murray et al., 2022; Oliveira-Tintino et al., 2023).

Efflux pumps are transmembrane proteins that play a significant role both physiologically and in multidrug resistance, by actively pumping antibiotics, biocides, and other substrates from the intracellular environment to the extracellular bacterial milieu. Thus, it is possible to highlight the six main families of efflux pumps: ATP-binding cassette (ABC) system, small multidrug resistance (SMR) family, multidrug and toxic compound extrusion (MATE) family, major facilitator superfamily (MFS), resistance-nodulation-division (RND) family, and proteobacterial antimicrobial compound efflux (PACE) family (Morais Oliveira-Tintino et al., 2022; Gaurav et al., 2023; Guo et al., 2020).

Among the efflux pump families, the MFS and MATE families are notable. These families express the respective proteins NorA and MepA, which are examples of efflux pumps involved in the extrusion of specific antibiotics (Morais Oliveira-Tintino et al., 2022; Henderson et al., 2021). Consequently, researchers increasingly seek to improve or develop medications that are effective in inhibiting efflux pumps. In this context, the search for bioactive compounds found in medicinal plants becomes important, as studies with natural products have already demonstrated their action against resistant bacteria, highlighting the bioactivity of sesquiterpenes (Moura et al., 2021; Dias et al., 2022; Parrino et al., 2020; Santana et al., 2023).

Sesquiterpenes are a subgroup of terpenes (one of the main groups of secondary metabolites produced by plants), consisting of three isoprene units – C15H24. They are found in oils called essential oils, considered important functional natural substances for demonstrating bioactive properties, with up to 5000 molecules described to date (Yamaguchi, 2019; Abu-Izneid et al., 2020; Do et al., 2025).

Among these molecules, nootkatone ((4 R,4aS,6 R)-6-Isopropenyl-4,4a-dimethyl-4,4a,5,6,7,8-hexahydro-2(3 H)-naphthalenone) stands out, which is present in essential oils found in citrus fruits such as grapefruit (*Citrus paradisi* Macfad.), from the Rutaceae family. This sesquiterpene is widely used in the food, cosmetic, pharmaceutical, and agricultural industries for producing aromatic inputs due to its pleasant fragrance, therapeutic products, as well as insecticides and repellents (Li et al., 2021; Wang et al., 2022; Habotta et al., 2023). Nootkatone exhibits bioactivities documented in the literature, such as antibacterial properties (Oliveira-Tintino et al., 2023), anti-inflammatory (Dantas et al., 2020), anticancer (Jha et al., 2022), antioxidant, among others (Zhang et al., 2023).

Therefore, since the sesquiterpene nootkatone demonstrates potential antibacterial activity and already exhibits other biological activities reported in the literature, this study aimed to evaluate its antibacterial action and inhibition of the efflux pumps NorA and MepA in *S. aureus* strains, aiming to identify a potential agent in combating bacterial resistance.

2. Materials and methods

2.1. Bacterial strains used

The respective bacterial strains used were *S. aureus* ATCC 29213, *S. aureus* 1199 (wild-type expressing the NorA protein), and *S. aureus* K2068 (overexpressing MepA). These strains were provided by the Laboratory of Microbiology and Molecular Biology at the Regional University of Cariri, and were maintained in stock on Heart Infusion Agar (HIA, Difco) at 4°C. Prior to the experiments, these strains were cultured on HIA and incubated at 37 °C for 24 h.

2.2. Obtaining and preparation of the substances used

The sesquiterpene nootkatone, antibiotics (norfloxacin and ciprofloxacin), DNA intercalator, efflux pump substrate the ethidium bromide (EtBr), Carbonyl Cyanide 3-chlorophenylhydrazone (CCCP), and Phosphate Buffered Saline (PBS) were obtained from Sigma-Aldrich Co., Ltd. The antibiotics norfloxacin and ciprofloxacin were used as specific substrates for the NorA and MepA efflux pumps, respectively. Initially, the sesquiterpene and antibiotics were diluted in 500 μL of dimethyl sulfoxide (DMSO at 10 %), followed by dilution in 9265 μL of sterile distilled water, achieving a concentration of 1024 μg/mL. Ethidium bromide (EtBr) was diluted in 9765 μL of sterile distilled water (1024 μg/mL). CCCP was diluted in a solution of methanol and sterile distilled water (1:1), achieving the same concentration as the other substances. PBS was dissolved by adding one capsule to each 200 mL of sterile distilled water.

2.3. Evaluation of intrinsic antibacterial activity

The analysis of nootkatone's antibacterial activity was conducted by determining the Minimum Inhibitory Concentration (MIC) using the broth microdilution method. Initially, bacterial inocula were prepared by suspending the strains in sterile saline solution, aiming for a 0.5 McFarland standard (1.5×10^8 CFU/mL). 900 μL of Brain Heart Infusion (BHI) culture medium (10 %) and 100 μL of the inoculum were added to Eppendorf microtubes. Subsequently, 100 μL of this solution was dispensed into each well of a 96-well microtiter plate, followed by serial dilutions of 100 μL of nootkatone up to the penultimate well, with the last well serving as the growth control. The compound concentrations varied from 512 to 8 μg/mL. The assays were conducted in triplicate. The plates were incubated in a bacteriological incubator (Sanyo CO2 Incubator MCO-17AC) at 37 °C for 24 h. The results were read by adding 20 μL of resazurin (0.003 g/mL) and observing the color change. A blue color indicated the absence of bacterial growth, while a red/pink color indicated bacterial growth (Coutinho et al., 2008; Humphries et al., 2018).

2.4. Evaluation of the potential synergistic action of fluoroquinolones with nootkatone

Based on the results of the MIC determination, bacterial inoculum, BHI culture medium (10 %), and nootkatone at subinhibitory concentration (MIC/8) were prepared in microtubes. (MIC/8 = 128 μg/mL in *S. aureus* ATCC 29213, and in *S. aureus* 1199; MIC/8 = 25,37 μg/mL in *S. aureus* K2068. Then, 100 μL of the solution was dispensed into 96-well microtiter plates, followed by separate serial dilutions with norfloxacin at 1024 μg/mL. For the negative control, solutions containing only inoculum and BHI culture medium were prepared, followed by serial dilution with norfloxacin. For the positive control, the efflux pump inhibitor CCCP (at subinhibitory concentration MIC/8) was used. CCCP was used to ensure that the strain did not possess an efflux pump. As the test sample, nootkatone (MIC/8) was used. The compound was added to a solution containing 10 % inoculum and BHI culture medium, followed by dispensing this solution into a 96-well plate and performing microdilution with norfloxacin. The tests were conducted in triplicate, and results were read by adding 20 μL of resazurin (0.003 g/mL) and observing the color change. A blue color indicated the absence of bacterial growth, while a red/pink color indicated bacterial growth (Coutinho et al., 2008).

2.5. Evaluation of efflux pump inhibition by the combination with antibiotics and ethidium bromide

Based on the results of the direct MIC determination, bacterial inoculum, BHI culture medium (10 %), and nootkatone at subinhibitory concentration (MIC/8) were prepared in microtubes (MIC/8 = 128 μg/

mL in *S. aureus* ATCC 29213, and in *S. aureus* 1199; MIC/8 = 25,37 µg/mL in *S. aureus* K2068). Then, 100 µL of this solution was dispensed into 96-well microtiter plates, followed by separate serial dilutions of antibiotics and EtBr, both at a concentration of 1024 µg/mL. As negative controls, antibiotics (specific substrates) and EtBr alone were used. Norfloxacin was used against the *S. aureus* 1199 strain, and ciprofloxacin against the *S. aureus* K2068 strain. EtBr (a nonspecific substrate) was used for both strains carrying the NorA and MepA efflux pumps. In the negative controls, solutions containing only inoculum and BHI culture medium were prepared, followed by microdilution with antibiotic or EtBr. For the positive control, the standard efflux pump inhibitor CCCP (MIC/8) was added to a solution containing 10 % inoculum and BHI culture medium at MIC/8. This solution was then distributed into a 96-well plate followed by microdilution with antibiotic or EtBr. The tests were performed in triplicate, and the subsequent steps followed as described in Section 2.3 (Coutinho et al., 2008).

2.6. Evaluation of efflux pump inhibition by fluorescence measurement

For this experiment, the *S. aureus* 1199 strain was cultured on BHI Agar medium and incubated at 37 °C in a bacteriological incubator for 24 h prior to the assay. The inoculum preparation was done in PBS until achieving a 0.5 McFarland standard (1,5 $\times 10^8$ CFU/mL). Nootkatone was tested at concentrations of 128 µg/mL and 64 µg/mL. For the assay, the following groups were prepared: the blank containing only PBS with EtBr (2 µg/mL); the negative control containing bacterial inoculum with EtBr (2 µg/mL); the positive control prepared with CCCP (128 µg/mL and 64 µg/mL) + EtBr + bacterial inoculum. To verify the inhibition of the efflux pump by nootkatone, the test solution was prepared, consisting of nootkatone (128 µg/mL and 64 µg/mL) + EtBr + bacterial inoculum. After preparing the substances and the inoculum, 100 µL of the inoculum was added to each well of a black 96-well culture plate (SPL Culture Plate 96 wells), and subsequently, 100 µL of either nootkatone, CCCP, or PBS was added. The plate was incubated for 1.5 h in a bacteriological incubator at 37 °C. Then, 100 µL of EtBr was added to each well, and the plates were further incubated for 1 h. The readings were performed using a Cytation 1 fluorescence microplate reader from BioTek® (Winooski, VT, USA) with Gen5™ 3.11 Software. Excitation was set at 530 nm and emission wavelength at 590 nm (Oliveira-Tintino et al., 2023).

2.7. Statistical analysis

The microbiological tests for determination of MIC and efflux pump inhibition were expressed as geometric mean and standard error of the mean. The remaining assays were expressed as arithmetic mean and standard error of the mean. The statistical analysis applied was One-way ANOVA, followed by the Bonferroni post hoc test. GraphPad Prism 5.0 software was used for analysis. All assays were performed in triplicate. Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Evaluation of direct antibacterial activity by MIC

It was found that nootkatone exhibited direct antibacterial action with an MIC of 203 µg/mL against the *S. aureus* K2068 strain. Against the *S. aureus* ATCC 29213 and *S. aureus* 1199 strains, nootkatone did not show clinically relevant antibacterial activity, demonstrating an MIC ≥ 1024 µg/mL (Table 1).

Table 1
Minimum Inhibitory concentration of Nootkatone against *S. aureus* ATCC 29213, *S. aureus* 1199, and *S. aureus* K2068.

	***S. aureus* ATCC 29213**	***S. aureus* 1199**	***S. aureus* K2068**
Nootkatone	≥ 1024 µg/mL ± 1	≥ 1024 µg/mL ± 1	203 µg/mL ± 1

The *S. aureus* K2068 strain exhibited a significantly lower minimum inhibitory concentration (MIC) for nootkatone compared to the reference strains ATCC 29213 and SA1199B, indicating greater susceptibility of this clinical isolate to the compound. This difference may be associated with specific genomic variations, including reduced expression or absence of efflux pumps such as NorA and MepA, which are widely described in *S. aureus* resistance to antibacterials (Kaatz et al., 1995; Costa et al., 2019). In the case of SA1199, for example, the expression of NorA contributes to reducing the effectiveness of several bioactive compounds, which may explain its higher MIC against nootkatone. Although ATCC 29213 is considered a standard susceptible strain, it did not show the same level of susceptibility observed in K2068, suggesting that additional factors such as membrane permeability alterations or metabolic differences may influence the compound's activity. These findings reinforce the potential of nootkatone as an antibacterial agent, particularly against multidrug-resistant clinical isolates, highlighting its applicability in the development of new adjuvants or alternative therapies (Tintino et al., 2018).

3.2. Evaluation of the potentiating action of fluoroquinolone by nootkatone

Against the *S. aureus* ATCC 29213 strain, in the combination of norfloxacin with nootkatone, the sesquiterpene at a concentration of 128 µg/mL reduced the MIC of the antibiotic by approximately 87.5 %, compared to the negative control (norfloxacin). The positive control (norfloxacin + CCCP) did not cause reduction in bacterial growth or reduction in the MIC of norfloxacin, indicating that the strain did not possess an efflux pump. Based on these results, the sesquiterpene nootkatone is potentiating the action of the antibiotic against the *S. aureus* strain (Fig. 1).

3.3. Inhibition of the NorA efflux pump assessed by reduction in antibiotic MIC and EtBr

Against the *S. aureus* 1199 strain in combination with norfloxacin, it was observed that nootkatone at a concentration of 128 µg/mL reduced the MIC by 92.1 % (Fig. 2A). In combination with EtBr, it showed a reduction of approximately 60.2 % in the MIC (Fig. 2B). Based on these

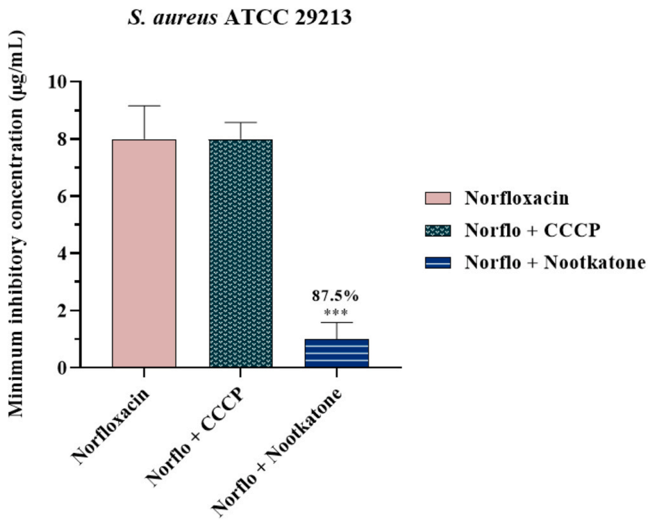

Fig. 1. Evaluation of fluoroquinolone potentiation by Nootkatone against *S. aureus* ATCC 29213. Nootkatone associated with norfloxacin. Norflo = norfloxacin; CCCP = Carbonyl cyanide 3-chlorophenylhydrazone; *** = $p < 0.001$ vs Norflo control, determined by One-way ANOVA followed by Bonferroni post hoc test.

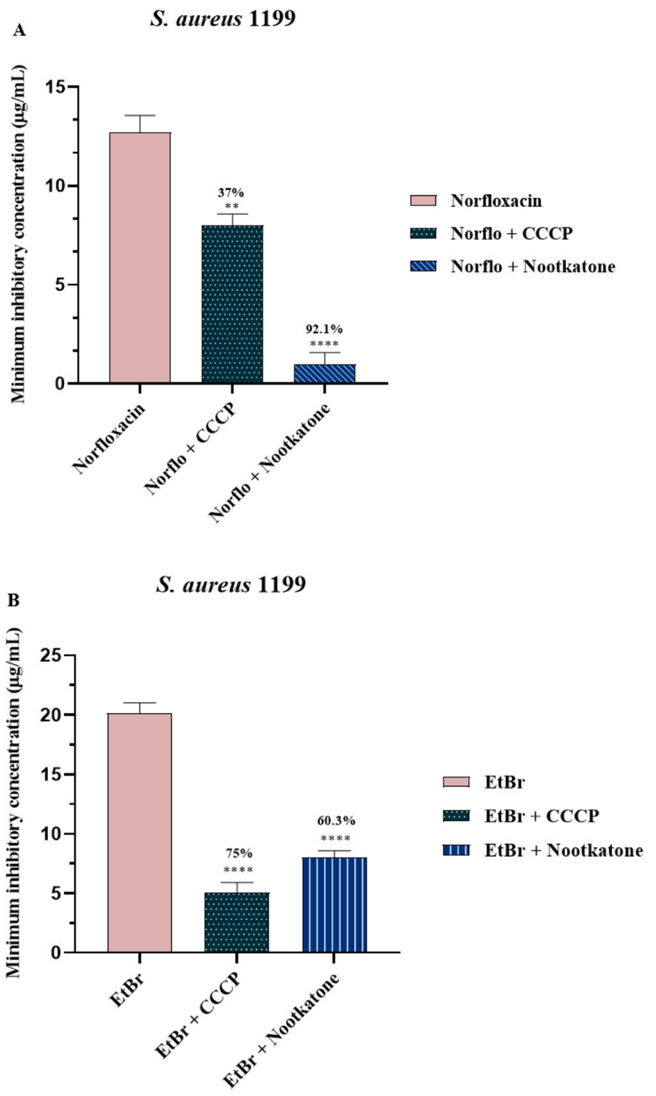

Fig. 2. Evaluation of NorA efflux pump inhibition against *S. aureus* 1199 strain by reducing the MIC of the antibiotic and Bret. Nootkatone associated with norfloxacin **(A)** and EtBr **(B)**. Norflo = norfloxacin; EtBr = ethidium bromide; CCCP = Carbonyl cyanide 3-chlorophenylhydrazone; **** $= p < 0.0001$ vs Norflo or EtBr control; ** $= p < 0.01$ vs Norflo control, determined by One-way ANOVA followed by Bonferroni post hoc test.

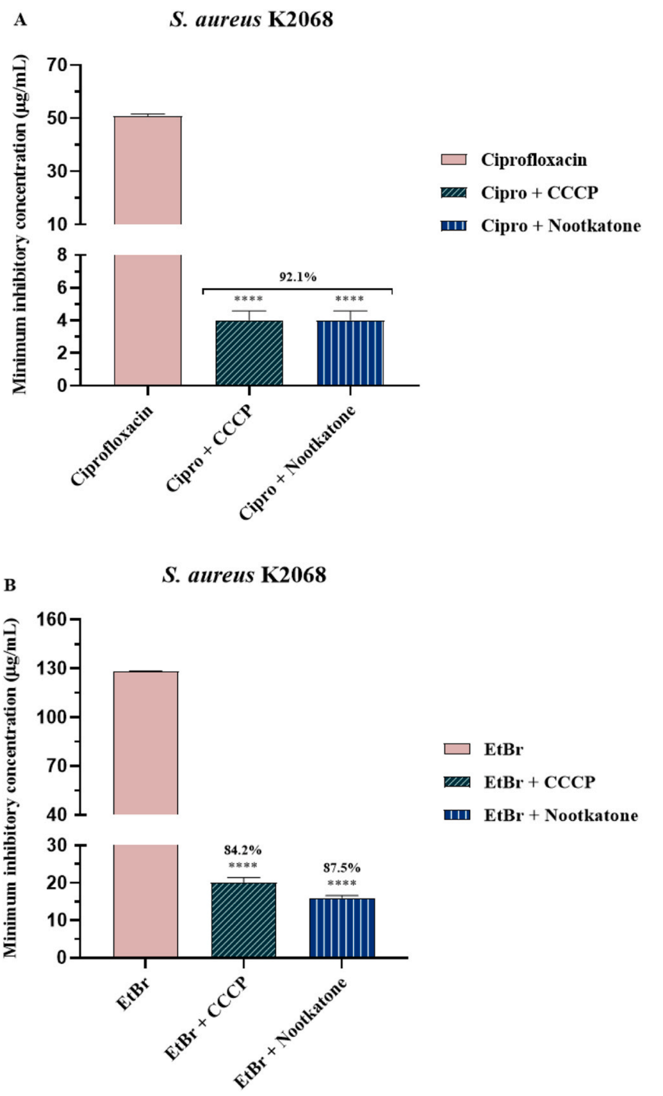

Fig. 3. Evaluation of MepA efflux pump inhibition against *S. aureus* K2068 strain by reducing the MIC of the antibiotic and Bret. Nootkatone associated with ciprofloxacin **(A)** and EtBr **(B)**. Cipro = ciprofloxacin; EtBr = ethidium bromide; CCCP = Carbonyl cyanide 3-chlorophenylhydrazone; **** $= p < 0.0001$ vs Cipro or EtBr control, determined by One-way ANOVA followed by Bonferroni post hoc test.

results, nootkatone potentiated the effect of norfloxacin and EtBr, potentially inhibiting the NorA efflux pump.

3.4. Inhibition of the MepA efflux pump assessed by reduction in antibiotic MIC and EtBr

Against the *S. aureus* K2068 strain, nootkatone associated with ciprofloxacin and the combination of ciprofloxacin with CCCP showed a reduction in MIC by 92.1 % for both (Fig. 3 A). When combined with EtBr, the MIC reduced by 87.5 % (Fig. 3B). These results possibly indicate inhibition of the MepA efflux pump by nootkatone.

3.5. Evaluation of efflux inhibition by fluorescence

In the evaluation of fluorescence emission in the *S. aureus* 1199 strain, it was observed that nootkatone at concentrations of 64 µg/mL and 128 µg/mL significantly increased fluorescence compared to the negative control (bacteria and EtBr). A significant increase in fluorescence can be observed in the positive control (CCCP + EtBr) compared to the negative control, confirming the reliability and reproducibility of the results (Fig. 4).

Studies have shown that the natural sesquiterpene, nootkatone, exhibited antibacterial activity against strains of *S. aureus* (GTC1181, 1186, 1770 and 97LSAP3), *Enterococcus faecalis* (NHp6), *Listeria monocytogenes* (GTC00149T) and *Corynebacterium diphtheriae* (GTC00263T). In the same study, nootkatone at a low concentration of 0.25 mM was effective in inhibiting biofilms in *S. aureus* strains (Yamaguchi, 2019). Studies indicated that nootkatone exhibited an MIC of 200 µg/mL against *S. aureus* strains (SJTUF 20758 and ATCC 25923) and also showed significant effects (at 50 and 100 µg/mL) in inhibiting biofilm formation in *S. aureus* (Farha et al., 2020).

Analysis of MIC by broth microdilution method with essential oil extracted from the peel of *Citrus maxima* (Burm.) Merr., which contains nootkatone as one of its constituents, showed antibacterial activity against Gram-positive strains of *S. aureus* (1096 µg/mL), *Bacillus subtilis* (1104 µg/mL), *B. licheniformis* (475 µg/mL) and *B. altitudinis* (1040 µg/mL) and Gram-negative strains of *Escherichia coli* (632 µg/mL) and *Pseudomonas aeruginosa* (720 µg/mL) (Chen et al., 2018). Nootkatone

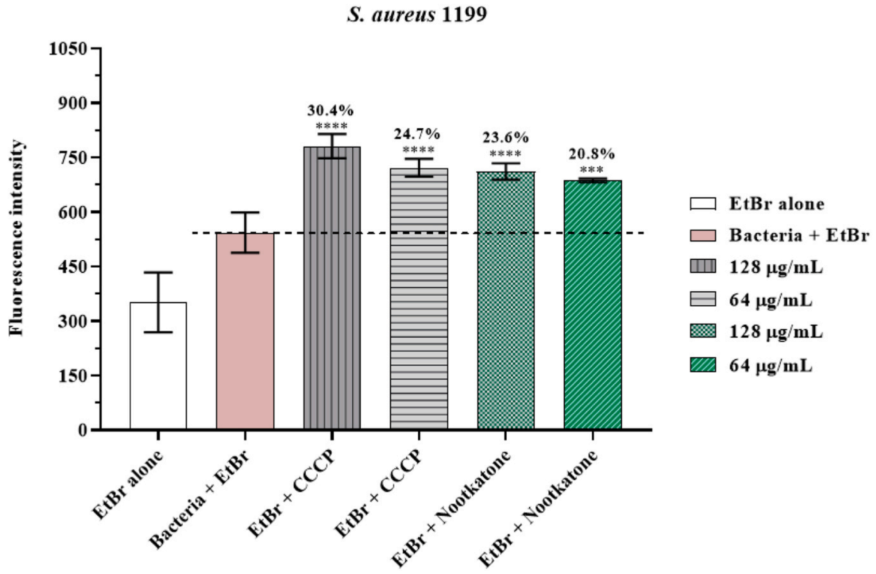

Fig. 4. Evaluation of Nootkatone in NorA efflux pump inhibition by fluorescence. Evaluated by fluorescence emission against the *S. aureus* 1199 strain carrying the NorA efflux pump. CCCP = Carbonyl cyanide 3-chlorophenylhydrazone; EtBr = ethidium bromide; **** = $p < 0.0001$ vs bacteria + EtBr; *** = $p < 0.001$ vs bacteria + EtBr, determined by One-way ANOVA followed by Bonferroni post hoc test.

isolated from the leaves of *Alpinia oxyphylla* Miq. at a concentration of 30 μg/mL inhibited the growth of multidrug-resistant bacteria including *E. coli, Klebsiella pneumoniae*, *S. aureus* and *Proteus vulgari*. The study demonstrated the significant susceptibility of MDR bacteria to nootkatone (Busayo et al., 2023).

The MIC of nootkatone was lower in *S. aureus* K2068 (expressing MepA) than in the *S. aureus* 1199 strain. It is important to highlight that *S. aureus* species are genetically highly variable. Even though strains belong to the same species, they can exhibit distinct genetic and phenotypic profiles. Therefore, it is likely that the *S. aureus* 1199 strain possesses additional resistance mechanisms that overcome the inhibitory effect observed at the MIC level against *S. aureus* K2068. It is also suggested that the resistance mechanism together with its phenotypic profile may not affect the action of nootkatone specifically (Lindsay, 2010; Wang et al., 2019; Michalik et al., 2025).

Studies conducted using the disc diffusion method with essential oils extracted from grapefruit peel (*Citrus paradisi*. L), which contains nootkatone as one of its main constituents, demonstrated inhibitory action against the growth of *E. coli* ATCC 25922 and methicillin-resistant *S. aureus* (MRSA) ATCC 25923 (El Houda et al., 2020) This study may partially support our findings by highlighting the direct activity of nootkatone against the *S. aureus* K2068 strain. Therefore, the use of nootkatone in bacterial control may offer multiple advantages, due to its ability to potentiate antibiotics in efflux pump-expressing strains. Thus, it can be inferred that nootkatone exhibits both direct antibacterial activity and indirect effects through the putative inhibition of NorA and MepA efflux pumps in resistant strains. These results also demonstrate that nootkatone may act on more than one target in addition to efflux pumps when combined with antibiotics, enhancing their action, as evidenced by the results obtained with the *S. aureus* ATCC strain.

Em fluorimetry assays, EtBr is an intercalating dye used as a parameter to assess efflux system inhibition. Due to its DNA intercalating properties, its fluorescence emission increases significantly upon binding to genetic material. The observation of increased fluorescence with this compound reinforces previous results, indicating an increase in EtBr concentration within the intracellular medium. Therefore, efflux pump inhibition is directly proportional to the fluorescence emission of EtBr (Blair; Piddock, 2016; Gibbons et al., 2003; Markham *et al.*, 1999). The present study demonstrated that nootkatone increased the fluorescence intensity of EtBr in the *S. aureus* 1199 strain, indicating inhibition of the NorA efflux pump and reinforcing previous microbiological results.

In studies using nootkatone to inhibit efflux pumps, this sesquiterpene showed potential inhibitory action against NorA and MepA when combined with antibiotics and EtBr. It was also observed an increase in EtBr fluorescence emission in the fluorimetry test in the groups treated with nootkatone (Oliveira-Tintino et al., 2023). These results corroborate the present study, indicating and reinforcing the significant action of nootkatone in inhibiting the NorA and MepA efflux systems.

It is also important to emphasize that recent studies have highlighted the promising role of natural, terpernes and sesquiterpenes as modulators of bacterial resistance, particularly through their interaction with efflux pump systems (Farha et al.,2020, Santana et al., 2023). For instance, compounds such as β-caryophyllene, isopulegol and farnesol have demonstrated inhibitory effects on multidrug resistance pumps in *Staphylococcus aureus*, thereby enhancing the activity of conventional antibiotics (Da Silva et al., 2024; Almeida-Bezerra et al., 2025; Santana et al., 2023). These findings reinforce the potential of sesquiterpenes as adjuvants in antibacterial therapy, supporting the relevance of our results with nootkatone. By situating our observations within this broader context, it becomes evident that nootkatone not only exhibits intrinsic antibacterial activity but also aligns with a growing body of evidence that natural sesquiterpenes can act as effective efflux pump inhibitors and antibiotic potentiators.

4. Conclusion

With the results of the present study, the sesquiterpene nootkatone demonstrated intrinsic antibacterial activity against the *S. aureus* strain K2068. Nootkatone has been shown to be a potential and putative inhibitor of the efflux pumps NorA and MepA expressed by the *S. aureus* strains 1199 and K2068, respectively, based on the results of MIC assays and fluorescence measurements with EtBr. Nootkatone also enhanced the antibiotic activity of fluoroquinolones. Therefore, further molecular biology or gene knockout studies are needed to better elucidate and prove the potential of nootkatone in its antibacterial action and inhibition of the efflux pump in *S. aureus* strains and to prove its mechanism of action.

CRediT authorship contribution statement

Daniel Sampaio Alves: Writing – original draft, Methodology, Investigation, Formal analysis. **Emílio Sousa Albuquerque:** Visualization, Validation, Methodology. **José Weverton Almeida-Bezerra:** Validation, Supervision, Resources, Data curation. **João Pedro Viana Rodrigues:** Visualization, Software, Methodology. **Roberto Nicolete:** Visualization, Validation, Supervision, Conceptualization. **Henrique Douglas Melo Coutinho:** Writing – review & editing, Supervision, Project administration. **Cícera Datiane de Morais Oliveira-Tintino:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Saulo Relison Tintino:** Methodology, Funding acquisition, Formal analysis. **Gabriel Gonçalves Alencar:** Writing – original draft, Methodology, Investigation. **Gustavo Miguel Siqueira:** Writing – original draft, Methodology, Investigation. **Gildênia Alves de Araújo:** Methodology, Investigation, Formal analysis. **Sheila Alves Gonçalves:** Writing – original draft, Investigation, Formal analysis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The authors are unable or have chosen not to specify which data has been used.

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